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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

EPIDEMIOLOGICAL EVALUATION OF MASTITIS CAUSED BY *STREPTOCOCCUS AGALACTIAE* AND *STREPTOCOCCUS UBERIS* IN FOUR DAIRY HERDS IN NORTH OF PORTUGAL

Marta Sofia Morais Vales

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This work was performed in collaboration with:

ABSTRACT

Bovine mastitis is one of the most prevalent and costly farm animals diseases that is widely spread all over the world, and a reason for concern for farmers and veterinarians, since it represents a great impact on cow welfare and in economic losses. Among all the pathogens that can cause mastitis, *Streptococcus agalactiae* and *Streptococcus uberis* are the most relevant. The main goal of this study was to analyze *S.agalactiae* and *S.uberis* isolates obtained from cows, that presented recurring mastitis infections. These cows were assayed at different time points in order to assess the molecular epidemiology of mastitis-causing pathogens.

Bacterial virulence genotypes were found to be associated with mammary infections. For this reason, several virulence markers were tested with the collected isolates. For the putative *S.agalactiae* isolates the following markers were tested: the fructose operons FO1 and FO3; the toxin production CAMP factor; and the gene responsible for the adherence to epithelium*, fbsB.* The results obtained showed that a large percentage of *S.agalactiae* isolates contained these virulence factors in their genome. For the putative *S.uberis* isolates the following markers were chosen for testing: the gene responsible for the adherence to epithelium in this species, *sua*; the bacteriocin nisin U; the plasminogen activator *pauA*; the toxin production factor CAMP; and an antimicrobial resistance gene *ermB.* With the exception for the CAMP factor, all these genes were shown to be present in the large majority of of *S.uberis* isolates assayed.

This work strengthens the hypothesis from previous studies concerning the high consistency and stability of these markers when used for identification of streptococci isolates. The obtained results suggested that there is a high prevalence of mastitis in the selected dairy herds, and that there are specific virulence patterns which are common amongst successful mastitis- causing pathogens. However, it was noticeable that pathogens with distinct virulence patterns were also able to cause recurring infections, meaning that infections apparently caused by atypical streptococci were also successful.

Keywords: Antibiotics resistance; Bovine mastitis; DNA markers, Dot-Blot hybridization; *Streptococcus agalactiae*; *Streptococcus uberis*; Epidemiology; Virulence factors.

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LIST OF ABBREVIATIONS

CAMP - Cyclic adenosine monophosphate

CMT- Californian milk test

DNA Deoxyribonucleic acid

fbsA- Fibrinogen-binding protein A

fbsB - Fibrinogen-binding protein B

LF- Lactoferrin

Fg – Fibrinogen

LGT- Lateral gene transfer

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GBS - Group B Streptococcus

opps - Oligopepide permeases

PCR - Polymerase Chain Reaction

PMN - Polymorphonuclear neutrophils

PTS- Phosphoenolpyruvate-dependent phosphotransferase system

rRNA - Ribosomal ribonucleic acid

SSC - Somatic cell count

Taq - DNA polymerase from *Thermus aquaticus*

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INTRODUCTION

1.Bovine Mastitis

Bovine mastitis is a disease that affects dairy herds worldwide and is a main concern for both farmers and veterinarians concerning animal welfare and economic losses. (Koskinen *et al*., 2009; Manthe, 2014; Reinoso *et al*., 2011; Taponen *et al*., 2009; Tomita *et al.,* 2007). The disease consists in an inflammation of the mammary gland mostly due to the invasion of pathogenic microorganisms through the teat canal. Any kind of trauma or irritants can damage the skin around the teat, which contributes to break the first line of defense against external pathogens (Almeida *et al.*, 2013). When pathogens enter the teat canal, they start to produce toxins, enzymes and surface proteins to promote adherence to the host tissue (Almeida *et al*., 2013). These aggressions cause an inflammatory response characterized by an increase of somatic cells count (SCC), with release of interleukins, polymorfonuclear neutrofiles (PMN), phagocytes and leukocytes to fight against this aggression. In case of inflammation, around 95% of cells present are PMN (Schukken, 2004).These inflammations interfere with the milk production and quality, reducing its economic value.

Depending on the range of clinical signs, mastitis can vary from subclinical to clinical presentation, which also depends on the virulence of the pathogens and the host´s immune system ability to respond to invasion (Erskine, 2001). Regarding the reservoirs of pathogenic agents, these microorganisms can be divided in two categories depending on their behavior: contagious, if the microorganism spreads from cow-to-cow through fomites namely dirty or spoiled teat cups, milker`s hands or even milking machines, and environmental, if the infection occurs due to ubiquitous organisms, which are found in the animal housing environment. (Reinoso *et al*., 2011). *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus dysagalactiae* and *Mycoplasm* are considered contagious agents, commonly causing longer and more prevalent infections. (McDonald,1979). *Streptococcus uberis, Escherichia coli, Klebsiella, spp* and *Enterobacter, spp* are considered environmental agents and usually cause more severe cases of clinical mastitis (McDonald, 1979). The above-mentioned bacteria belong to a group of major pathogens which cause the greatest milk alterations and economic losses. Coagulase-negative staphylococci and *Corynebacterium bovis* are examples of minor pathogens, usually not associated with large changes in milk parameters (Harmon, 1994). However recent studies suggest that the presence of these minor pathogens can increase the risk of intramammary infection by major pathogens (Reyer *et al*.,2012; Zadocks *et al*., 2001).

2.Streptococcus

Streptococcus are gram-positive bacteria with particular traits such as being catalase-negatives, facultative anaerobes and lactic acid producers. (Facklam,2002). These bacteria are usually commensal organism in mammalian skin membranes, such as the mucosa and skin surface. However, some behave as opportunistic bacteria and may cause infection if the host defense barrier is broken (Almeida *et al*, 2013, Porfírio, 2013).

Although many streptococci have been identified as mastitis causative agents, *Streptococcus agalactie* and *Streptococcus uberis* are generally regarded as the most significant pathogens (Jones and Bailey, 2009; McDonald *et al.,* 2005; Kuang *et al.,* 2009; Unnerstad *et al.,* 2009).

2.1. *Streptococcus agalactiae*

Streptococcus agalactiae is a pyogenic and contagious agent which causes mostly subclinic and also mild to moderate mastitis in ruminants. In general, cows infected with this pathogen present more than one affected quarter. These bacteria do not survive for long periods in the environment, nevertheless they can persist for undetermined time in mammary glands, if good practices are not implemented (Harmon,1994; Martinez *et al*., 2000; Ruegg,2005; Wilson *et al.,*1997).

Streptococcus agalactiae belongs to Group B Streptococcus (GBS), which is a Lancefield´s classification for hemolytic streptococci. (Radtke *et al*.,2010). These opportunistic bacteria are able to cause disease in humans, both adults and children preferentially in pregnant women, diabetics, older people or immunocompromised patients. *S.agalactiae,* which is commonly found in genitourinary and gastrointestinal tracts, could also cause septicemia, meningitis and pneumonia in neonates, breast abscess formation and endocarditis in women, and soft tissue and osteoarticular infections in elderly adults (Glaser *et al.*,2002; Haguenoer *et al.,*2011; Radtke *et al*., 2010; Zadocks & Fitzpatrick, 2009; Zadocks *et al.*,2011).

Several epidemiological and molecular studies have been conducted to analyze the persistence, transmission routes and reservoirs of human and cattle strains. Two hypotheses for the relation between these two groups of strains have been proposed, one postulates that, there is a direct transmission between cattle and humans and the other states that human strains evolved from bovine sources (Zadocks *et al*. 2011).

In herds with a good health management plan, it is possible to completely eliminate *Streptococcus agalactiae* through the implementation of specific measures, namely prevent the spread during milking, by avoiding milking the infected cows first, and perform dry-cow therapy.

Additionally, these infections can be permanently eliminated with intramammary or injectable therapy (Keefe,1997).

Infections cause by *S.agalactiae* decrease milk production and modify their parameters. In fact, a milk free of mastitis must have bellow 200x10e^3 somatic cells per milliliter, and any increase of SCC in the bulk tank lead to pecuniary penalizations for the farmers (Jones & Bailey, 2009).

2.2.*Streptococcus uberis*

Streptococcus uberis is one of the most important causative agents of mastitis all over the world. As an environmental pathogen, *S.uberis* are spread to uninfected cows throuhgt their contact with contaminated surfaces in the animal housing (Peterson-Wolfe & Currin, 2012; Pryor *et al*.,2009; Tassi *et al*.,2013; Zadocks *et al*.,2001; Zadocks *et al*., 2005).

This pathogen causes subclinical and clinical mastitis in lactating and non-lactating cows, with more emphasis in the dry period, calving and early lactation. There is an increased risk for new infections in the dry period and for cows in the lactating early period due to stress and postpartum low immunity (Oliver *et al*., 2004; Peterson-Wolfe & Currin, 2012; Zadocks *et al*., 2003). Due to its ubiquitous nature, *S. uberis* is a very prevalent species, however the knowledge about its epidemiology is still incomplete (Phuektes *et al*., 2001; Reinoso *et al.,* 2011; Zadock *et al.*, 2003).

These bacteria can be found in many surfaces such as the bovine udder, excrements or bedding, therefore maintaining a clean and dry environment is very important to prevent the infection. Additionally chronic subclinical mastitis infection could also serve as a reservoir of contamination for healthy cows (Peterson-Wolfe & Currin, 2012; Zadocks *et al.,* 2003), and many studies report that in some herds cow-to-cow transmission can be a vector of infection (Phuektes *et al*.,2001;McDougall *et al*., 2004; Zadocks *et al.,* 2001). However in the animals which are not milked, like dry cows and preparturient heifers, this kind of transmission cannot be considered, making the environment the most likely source of infection (Zadocks *et al.,* 2003).

Bacterial typing studies revealed high serologically and biochemically heterogeneity in *S. uberis* (Zadocks & Fitzpatrick, 2009).Generally the persistence of infection and treatment failures can be attributed to adherence and invasion factors and and to this capacity to survive in mammary epithelial cells (Bentley *et al.,* 1993; Zadocks & Fitzpatrick, 2009).

Regarding treatment, long lasting antibiotics are nowadays widely used to control the risk of mastitis. Nevertheless, some studies refer that the use of an internal teat sealant and antibiotics treatments at dry off period could reduce the rate of new infections (Peterson-Wolfe & Currin, 2012).

Application of good management practices in herds have been successfully employed in order to reduce the effect of contagious agents, however, their success is limited towards controlling mainly environmental agents like *Streptococcus uberis* (Pitkälä *et al.*, 2008; Tomita *et al.,* 2007).

3.Mastitis Resistance

To prevent possible entries from pathogens, cow´s udders have some strategies of defense. The first one, which prevents the entry of pathogenic agents in the mammary gland, is the teat canal that provides a physical barrier and the sphincter muscle (within the teat canal) that helps to expel out this microorganisms through washing-out effect of milking and epithelial desquamation (Sandholm *et al.,* 1995). Studies show that a higher ratio of milk flow leads to less probability of mastitis infections, mostly in the case staphylococcal and streptococcal infections. The size of the teat canal and strength of the sphincter muscle are crucial to prevent the bacteria from reaching the mammary gland cistern. The medium diameter of the teat canal is between 2.3 and 5mm, therefore there is an increased risk of mastitis in older cows as they have more dilated teat canals. Shorter teats milked faster and fuller and avoiding residual milk, therefore, decreasing the susceptibility to infection. Since the conformation of udders is heritable, and that deep and pendulous udders have more predisposition to higher SCC and mastitis (Seykora & McDaniel, 1985), extra care must be taken at the end of milking because the teat canal remain open for at least two hours, which contributes to an increase of ascending infections (Jones & Bailey, 2009; Sharif *et al*.,2009).

Keratin, a fibrous protein with bacteriostatic properties, is present in lining cells of the teat, which greatly contributes to slow the possible progression of pathogens. Therefore, trauma or lacerations in the teat must be avoided, as they damage the cells producing keratin and mucous, which increases the risk of bacterial colonization (Jones & Bailey, 2009). One study found that there is about three times more prevalence of new infections by *Streptococcus agalactiae* in quarters with acute teat end lesions than in healthy or small lesion quarters. A well planned milking is a good practice in order to avoid teat trauma and further hyperkeratosis. Moreover, care should be taken, to not milk a dry quarter, as in addition to the improper stimulation of the animal, overmilking can predispose to erosion of the orifice and cause severe lesions which may lead to persistent subclinical mastitis (Neijenhuis *et al.,* 2001; Seykora & McDaniel, 1985). The second line of defense, consists in the immunological properties of milk, which is not very favorable to the growth of bacteria.

Genetics factors, udder health and lactation stage can interfere in the concentrations of important antibacterial enzymes and other molecules present in milk such as lactoperoxidase, lysozyme, lactoferrin, transferrin, immunological defense mechanisms and the complement

system (Sandoholm *et al.,*1995). In case of inflammation, many mediators migrate through circulatory system to quell the infection, such as prostaglandins, leukotrienes, interleukines (IL17), lympocytes, neutrophyles, phagocytes,PMN leukocytes, major histocompatibility class I and II and toll-like receptors (Tassi *et al.,* 2013; Zadocks & Fitzpatrick, 2009). However it should be noted that PMN leukocytes and phagocytes are attracted in a wide number to milk which causes an increase of somatic cells count (SCC). Moreover, substances released by PMN destroy the alveolar structure that upon healing turns into cicatrization tissue, which can be problematic for antibiotic treatment (Jones & Bailey,2009).

Specific cow characteristics influence the onset of mastitis and increase the duration of clinical mastitis namely genetics, parity,age, negative energetic balance, immune status, nutritional status, deficiencies of some components of diet like selenium, E vitamin, α or β carotene (Abureema et al., 2014; Hagnestam-Nielsen *et al*., 2008; Harmon,1994; Zadocks *et al.,*2001).

Lactoferrin and transferrin are two important iron binding proteins that are part of antibacterial defense, preventing bacterial growth by competition against bacterial iron uptake. In case of mastitis, their concentration in milk increases considerably. Lactoperoxidases are enzymes responsible for the oxidation of bacterial cell wall componentes. These enzymes are regulated by the concentration of hydrogen peroxide which formation is inhibited by the low oxygen pressure in case of mastitis.

Lysozyme is an antimicrobial enzyme, that regardless their small concentration in milk, causes the hydrolysis of the bacterial cel wall causing the osmotic lysis of bacteria (Sandholm *et al.,* 1995).

Altogether these studies show that in case of infection, milk composition is altered. An increase in vessels permeability and SCC and, a decrease of some components such as lactose, α-lactalbumin, fat, casein and calcium translate in an impairment of milk quality (Harmon, 1994).

4.Bacterial tools to successfully infect the host

From the moment bacteria invades the teat canal and reach the milk cistern, a complex process of adaptation is started. In general, when the udder´s defense is broken this is due to several bacteria species. These bacteria compete with each other seeking dominance to successfully infect the host. To accomplish this task, many virulence factors are induced in order to enhance the host invasion, i.e., to resist to the immune response from the host; to grown in milk; to adhere to the mammary epithelial cells; and to resist phagocytosis (Pryor *et al*.,2009). Therefore, an in depth analysis of virulence factors allows for an enhanced understanding of the adaptation of *S.agalactiae* and *S.uberis* in the mammary gland.

Bacteria with a good adherence and invasion capacity are more fit to ensure their spread in the host, therefore their virulence aptness is increased when these factors are present (Sandholm et al., 1995). Not surprisingly, bacteria production of surface adhesins, like fibrinogen, is extremely valuable at the early stages of infection, as they allow to counteract the flushing effect during milking or the phagocytic action, both of which, constitute barriers to bacterial propagation (Patti et al., 1994).

4.1 Invasiveness patterns

In *S.agalactiae*, several families of fibrinogen binding proteins (Fb) have been studied, namely the fibrinogen binding protein A (FbsA) and fibrinogen binding protein B (FbsB) (Devi et al., 2010; Porfírio, 2013). FbsA promotes binding to the human Fb, and FbsB has a terminal region that binds only in bovine Fb. Binding is increased by calcium, which is present in milk (Devi *et al*., 2010; Jacobsson *et al*., 2002; Porfírio,2013).

In *S. uberis*, Sua is an adhesion molecule with high affinity to lactoferrin (Almeida et al., 2006). Lactoferrin (LF) is an iron binding glycoprotein present in milk, known for their bacteriostatic, bactericidal, antifungal, antiviral, antitumural and immunomodulating properties, which takes part in the innate immune response. LF is synthesized by glandular epithelium cells in the mammary gland and studies show that infected quarters present higher concentrations of LF. Additionally, it is reported that, *S uberis* can induce LF synthesis in milk enhancing its resistance to lactoferrin activity, which confers an advantage over competing bacteria (Chaneton *et al.,* 2008).

Pathogens with an hyaluronic capsule, are protected from host defence to some extent, because neither antibodies or complement factors can attach to the bacteria surface and perform the recognition before phagocytes (Sandholm *et al*., 1995; Ward et al.,2001). Three genes are involved in the production of a hyaluronic acid capsule: *hasA, hasB* and *hasC*. (Ward et al., 2001). In *S.uberis*, the capsule production requires the presence of both *hasA* and *hasC* genes (Field et al., 2003), however, studies refer that in *S.uberis* the presence of an hyaluronic acid capsule is not essential for a successful infection (Ward et al., 2009).

4.2- Virulence patterns

Streptococcus agalactiae has the ability to quickly adapt to adverse conditions in the host, such as osmolarity, pH, starvation, temperature variations and oxidative stress, through the synthesis of proteins that act as proteases and chaperones.

For their growth, bacteria need to import some carbon sources, and in *S. agalactiae* the sugar specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) enzyme II

complexes like lactose, fructose, glucose or mannose can be used for this purpose (Glaser et al., 2002).

In the absence of the primary source of carbon (carbohyrdrates), lactose and fructose can help to supply energy, however, only bovine strains present this fructose utilization pathway. In the utilization of fructose, a four-gene operon is involved: the phosphotransferase system fructospecific IIA componente (*fruD*), the fructose-specific IIBC PTS component (*fruC*), the fructose-1-phosphate kinase (*fruP*) and a transcriptional regulator (*fruR*) (Richards et al., 2011). The fructose and lactose operons in *S.agalactiae* display high sequence homology (99%) with the ones present in other bovine mastitis causative bacteria, which suggests that lateral gene transfer (LGT) between different species can occur in the udder (Zadocks et al., 2011).

Extracellular proteins have been acknowledged as virulence factors, due to their ability to bind host proteins and provide resistance against reactive oxygen species produced by phagocytic cells. These proteins also allow bacterial growth, due to their capacity to break down glucose as a carbon source and to obtain energy, in addition to conferring protection to the host immune system (Holzmuller et al.,2006; Madureira et al.,2007; Pancholi & Fischetti, 1992). The glyceraldehyde-3-phosphate dehydrogenase, *(GAPDH*) is one of those proteins and is present both in *S. uberis* and *S.agalactiae* (Ling et al., 2004; Maeda et al., 2004; Reinoso et al., 2011).
Concerning toxin production, the CAMP factor is one of the most studied in *S.agalactiae* and *S.uberis.* The CAMP factor is a pore forming protein, which can cause the phospholipidic hydrolysis of the red blood cells membrane and lead to cell lysis (Lang & Palmer, 2003).

S.uberis is unable to grow in milk, unless is able to hydrolyze host proteins. Therefore, *S.uberis* uses a caseinolytic enzyme (plasmin) in order to acquire the essential nutrients necessary for growth. The extracelular plasminogen activator (PauA), hydrolyses plasminogen to plasmin allowing the release of amino essential acids and peptides from host proteins (Leigh et al., 1993; Smith et al., 2002). This means that bacterial growth in milk might not be possible without an intact oligopeptide transport system, whose function is the acquisition and accumulation of amino acids within the bacteria. These bacterial oligopeptide permeases (opps) include five proteins: oppA, oppB, oppC, oppD and oppF. In particular, oppF seems to play an important role in providing energy for peptide substrate transportation, allowing the growth of *S.uberis* in milk (Smith et al., 2002; Reinoso et al., 2011).

Bacteriocins are antimicrobial peptides produced by bacteria in response to direct competition between strains, which are able to inhibit the growth of the same or related species. Uberolysin and nisin U are the most studied bacteriocins (Wirawan et al., 2006). Studies have shown that strains that produce nisin U, are usually predominant in case of infection showing that the production of nisin U leads to a competitive advantage in intramammary infection (Pryor

et al., 2009). Paradoxally,only a few strains of *S.uberis* seem to have the capacity to produce nisin (Wirawan *et al*., 2006).

5.Mastitis control programs

Bovine mastitis is a major cause for concern among veterinarians and producers because it affects both the quality and the quantity of milk produced by dairy herds (Giraudo *et al.,* 1997). New mastitis control programs have been implemented in herds, which includes antibiotic and prophylactic therapy, postmilking teat disinfection and culling of chronically infected cows. These programs have been very successful in controlling cases of contagious pathogens like *Streptococcus agalactiae;* however the application of these measures are less effective regarding the control of environmental pathogens, such as *Streptococcus uberis* (Phuektes *et al*., 2001;Oliver *et al*., 2004).

It is very important that livestock producers receive specialized training in order to better understand and apply all the required preventive measures (Cerqueira *et al*., 2011).

The Californian Milk Test (CMT) is one of the most commonly used methods to evaluate subclinical mastitis by providing an estimate of somatic cells present in milk samples (Kuang *et al.,* 2009; Pyorälä, 2003). The reaction in which the test is based consists in the interaction between the somatic cells resultant from an inflammatory response, and reagent/detergent (usually Bromocresol) together with a pH indicator. In case of subclinical mastitis, a higher somatic cells count in the sample, promote the formation of a gel in CMT racket.

This test is very useful because, besides to be very easy to perform, it allows the farmer to identify the risk animals and collect their milk samples for further analyses, thus contributing to improve herd control programs in management and animal health (Brito *et al.,* 1997).

Other preventive measures can also be taken by farmers namely avoiding the entry of flies in the barn, since these insects can act as a disease transmission sources. Moreover, so some attention must be given to humid and cool places, carry out animal segregation during milking to reduce the contact between ill and healthy cows (Sharif *et al*., 2009; Zadocks, *et al.,* 2001) and perform periodic maintenance to milking machine in order to avoid vacuum fluctuations and overmilking (Giraudo *et al*. 1997; McDonald, 1979).

Treatment with antibiotics is widely spread in farms, and some studies show that antimicrobial treatments reduce the SCC and present higher bacterial cure rates and lower the risk of premature culling when administered intramuscularly compared to intramammary application (Pantoja et al.,2009; Sandgren *et al*., 2007).The supervision of a veterinary during all drug treatments is crucial for the success of antibiotics treatment as an efficient control measure (Rajala-Schultz *et al.,* 1999). However, proper identification of pathogens is required to correctly

apply the most suitable drug, taking into account resistance and susceptibility to antibiotics by specific pathogens (Pitkälä *et al.,* 2008).

Efficient treatment of clinical mastitis depends on several factors that the veterinarian must be aware, namely, the cow traits (age, state of lactation, immune response, SCC), the pathogen (pathogenicity, response to therapy and virulence factors), and drugs (spectrum, route, concentration and duration). Therefore a better understanding of all these factors increases the success of treatments (Barkema *et al.,*2006; Pinzón-Sanchez & Ruegg, 2011).

Concerning resistance to antibiotics, several studies have reported that resistance to gentamicin, erytromicin, pirlimycin and tetracycline are the most common (Minst *et al.*, 2012). In steptococci, the most usual macrolide resistance mechanism is a modification in ribosomes mediated by a methylase that is encoded by an *erm* gene (mainly *ermB*), which confers resistance to erythromicin and inducible resistance to streptogramin B and lincosamins (Duarte *et al*.,2004; Roberts *et al.,* 2002; Weiseblum *et al.,* 1985). Some streptocci are multidrug resistant, but resistance rates are lower in *S.agalactiae* than in *S.uberis*. Farms with more than eigthy cows have more apparent antibiotic resistance. A good choice for treatment appears to be penicillin and ampicillin due to is lack of resistance in herds, so β-lactamics remain the drugs of choice for treatment of these pathogens (Minst *et al*., 2012).

The application of the five point plan mastitis control program allowed for a rapid progress in reducing subclinical and clinical mastitis in herds all over the world (Barkema *et al.,* 2006; Fernández *et al.,* 2013).

6. The role of molecular biology in veterinary epidemiology

Molecular epidemiology is an interdisciplinary approach of paramount importance in order to ascertain causes, patterns, distribution and effects of infectious or non-infectious diseases in populations, either human or animal, using molecular biology methods and encompassing disciplines as molecular biology, population genetics and epidemiology (Foxmann & Riley, 2001; Riley, 2004).

In recent years bioinformatics tools are being widely used in molecular epidemiology, allowing to process, analyze and organized efficiently any kind of molecular typing data. One of the greatest advantages of molecular tools is the ability to process a large number of strains or to analyze simultaneously several loci while generating unequivocal data that can be stored and shared for further studies. Additionally, the integration of all this information can be used to generate epidemiological models that could contribute to understand epidemiological factors of persistent pathogens and their behavior through space and time (Muellner *et al*., 2011; Archie *et al*., 2009).

As mentioned above, bovine mastitis can be caused by either contagious or environmental pathogens, *Streptococcus agalactiae* and *Streptococcus uberis* respectively, and it is essential for the veterinarian to understand the diversity and behavior of these species, in order to develop effective control measures and conduct a good prophylaxis to prevent future infections. In this regard, molecular studies are invaluable to characterize differences between and within infectious bacterial species, and to unveil virulence factors, transmission and adapting mechanisms (Muellner *et al.,* 2011; Rato *et al*., 2008). The data are particular helpful to assist veterinarians overcoming important barriers encountered in clinical practice, as it is the efficient use of antibiotics (Frye et al., 2000)

7. Main goals to achieve

The main objectives of this project were:

- To characterize important virulence markers in *S.agalactiae* and *S.uberis*.
- To test the markers previously characterized in new isolates and confirm the presence of important virulence factors.
- To monitored selected animals previously known to be positive to *Streptococcus agalactiae* or *Streptococcus uberis* and evaluate the taxonomic and genotyping identity of reisolates that might reveal possible differences in adaptation and persistence of strains.
- To gather as much clinical information as possible from the infected animals in order to correlate with disease prevalence and with bacterial lineages that might contribute to unveil informative epidemiological disease patterns.

MATERIALS AND METHODS

1. Milk samples and bacterial isolates

For this study four dairy herds in an intensive system were selected, two of them had mostly isolates of *S.agalactiae* (Barcelos and Póvoa do Varzim), while the other two had *Streptococcus uberis* (Barcelos and Maia).

From November 2013 to April 2014, herds were visited once a month, with occasional exceptions, to monitor and evaluate the morning milking. The general state of lactation cows was evaluated, CMT was performed (Brito *et al.,* 1997) to determine the SSC. A numeric scale was used for SSC: 0 to normal milk, 1 to medium SSC and 2 to a severe SSC. Lactating cows were also screened for signs of clinical mastitis and a dichotomous scoring alphanumeric

system was used to classify the general state of cows and milk features: 1=abnormal milk, 2 = abnormal milk plus inflamed quarter, 3 = abnormal milk plus inflamed quarter and systemic illness and a letter A = milk with flakes, B = presence of clots, C = watery discharge and D= bloody discharge (Shukken & Welcome, 2004).

Before collecting the milk samples the teat ends were disinfected with cotton soaked in alcohol (70 %) and around 20 ml of milk were collected (5ml from each quarter for composed samples, or 20 ml from a specif quarter in follow-up analysis). All the samples were sent to SEGALAB (Laboratório de Sanidade Animal e Segurança Alimentar, S.A), to perform the counting of somatic cells and to identify bacterial isolates using the VITEK 2 system (bioMérieux, Durham, NC).

During the mentioned period and for this work a total of 233 bacterial isolates were obtained, 158 from *S. agalactiae* end 75 from *S. uberis*. As the central objective of this work was to study the bacterial isolates from the same infected cows at different time points, special emphasis was placed on the analysis of these isolates.

Five strains listed in Table I and previously characterized by Almeida et al., 2013 were used as controls.

Table I - Bacterial strains used as controls (Almeida *et al.*, 2013)

Strain	Species	Location	Source
SA A9	*Streptococcus agalactie* LMG 15083	-	LMG
SA7	*Streptococcus agalactie*	Vila do Conde	SEGALAB
SUA12	*Streptococcus uberis* LMG 9465	-	LMG
SU3	*Streptococcus uberis*	Barcelos	SEGALAB
SAUR1	*Staphylococcus aureus* LMG 8224	-	LMG

LMG- Belgian Co-ordinated collections of microorganisms, Gent, Belgium.

2. Bacterial culture conditions and DNA extraction

All the samples identified as *S.agalactiae* or *S. uberis* by the VITEK 2 system were cultured in Brain Heart Infusion (BHI) (biolab®, Hungary) at 37ºC. After growth in BHI medium, all isolates were stores at -80ºC in 20% glycerol.

DNA was extracted from pure cultures using the E.Z.N.A bacterial DNA purification Kit (Omega Bio Tek, Norcross, GA), following the manufacturer's instructions. The Qubit 2.0 Flurometer HS Assay (Invitrogen, Carlsbad, CA) was used to quantify the extracted DNA, and the quality was assessed by electrophoresis using 1% agarose gels stained with GelRed (Biotum). Gel images were obtained using a Gel-Doc system (Bio Rad).

3. PCR amplification

For amplification of the *cfb* (CAMP factor) and *pauA* genes, primer-pairs were designed using the Vector NTI software (Invitrogen, Carlsbad,CA.) and synthetized by STABVida (Lisbon, Portugal). Amplicon specificity was confirmed using the BLAST software (Basic Local Alignment Search Tool) (Altschul,1990) (BLAST, http://blast.ncbi.nlm.nih.gov/).

The PCR master mix contained 1x Dream Taq buffer, containing 1.5mM of $MgCl_2$ (Fermentas, Ontario, Canada), 0.2mM of each dNTP (Fermentas), 0.2µM of each primers (forward and reverse) and 1U of DreamTaq DNA polymerase (Fermentas). 25ng of bacterial DNA from pure bacterial cultures were used in each reaction. The PCR conditions were as follows: an initial denaturation of 95ºC for 5 min, 35 cycles at 90ºC for 30s, 55ºC for 30s, 72ºC for 45s, followed by a final extension of 10min at a temperature of 72ºC.

PCR products were visualized in 1.5% agarose gels stained with 3µl of GelRed (Biotum®) the expected bands were cut using a scalpel and purified using the Ilustra® GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). To confirm their identity these amplicons were sequenced by STABVida (Lisbon, Portugal).

Table II- Taxa specific and functional markers and PCR primers sequences used in this study.

Molecular markers	Primers sequence (5'-3')		T_a	Amplicon Lenght	Target gene	Reference
F1-SAA9	Fwd	TTATGCTCGTCTTGCTCTTTACGG	56ºC	285 bp	-	Almeida et al., (2013)
	Rev	GCACACGTCCAAGTGATGTAGCTG				
F1	Fwd	TTATGCTCGTCTTGCTCTTTACGG	54,6ºC	285 bp	-	Almeida et al., (2013)
	Rev	GCACACGTCCAAGTGATGTAGCTG				
SU	Fwd	TCGTTTGTATACGCTTGARGCT	50,6ºC	229 bp	-	Almeida et al., (2013)
	Rev	CACGTCTCTATAAAAGGAATTCCC				
A1	Fwd	ATGTAGCTGCTGATTCTGTCATAA	52,6ºC	314 bp	-	Almeida et al., (2013)
	Rev	AATAGCTGGTGTAGATTTGACTGC				
sua	Fwd	TCAGTTGTTGTGATTGCTGACGTC	56,0ºC	600 bp	*sua*	Porfírio,2013
	Rev	CAAACAAGTGGTTTCAGGTCCATT				
fbsB	Fwd	ACAAAGTTCAGTTGCGCAAACT	52,9ºC	525 bp	*fbsB*	Porfírio,2013
	Rev	CGCGATGAGATTGATTTACTCA				
FO1	Fwd	TCCACCACGTTATTGAGAGTTT	50,6ºC	331 bp	*fruR*	Richards et al., (2011)
	Rev	TCTCAATTTCTTCGATCTCATGTGC				
FO3	Fwd	TCTCAATTTCTTCGATCTCATGTGC	52,6ºC	348 bp	*fruD*	Richards et al., (2011)
	Rev	CAGGTCTTGTTGTCGAAAACGATTA				
NU1	Fwd	CCAAGGTTGCAGCGCATTT	51,5ºC	331 bp	*nsuR*	Richards et al., (2011)
	Rev	CCCCTTATTGTCTTGATGGGATT				
NU3	Fwd	AATCAAATCGTTGATGAAAATGACC	50,6ºC	502 bp	*nsuF*	Richards et al., (2011)
	Rev	AAACTTCTCCGTAATCCCAAACTTC				
V1	Fwd	TGCTTGGTGACGATTTGATG	58,0ºC	300 bp	*hasC*	Ward et al., (2001)
	Rev	GTCCAATGATAGCAAGGTACAC				

V2	Fwd GCTCCTGGTGGAGATGATGT Rev GTCACCAGTGTAAGCGTGGA	55,0ºC	189 bp	*gapC*	Reinoso et al., (2011)
V3	Fwd GGCCTAACCAAAACGAAACA Rev GGCTCTGGAATTGCTGAAAG	54,0ºC	419 bp	*oppF*	Smith et al., (2002)
CAMP	Fwd GGATTCAACTGAACTCCAACAGCA Rev CATGCTGATCAAGTGACAACTCCA	57,0ºC	614 bp	*cfb/cfu*	This study
pauA	Fwd TTTTGGGAATATTTGGTTGTGC Rev TCAACCCGTTTTCTGAGAATAA	55,0ºC	427 bp	*pauA*	This study

4. Dot-Blot screening

Purified PCR products were labelled with digoxigenin to obtain DNA probes, using the DIG-High Prime labelling kit (Roche, Basel, Switzerland) according to the manufacturer´s instructions. For marker F1, a family-specific marker previously validated (Almeida *et al*., 2013), a new probe was obtained using as template DNA from *S. agalactiae* SAA9.

To perform the Dot-Blot hybridization 100ng of heat-denatured DNA from each bacterial strain was spotted into a nylon membrane optimized for the transfer of nucleic acids (Amersham Hybond™-N GE Healthcare, Buckimghamshire,UK), using a Bio-Dot apparatus (Bio Rad) (Tables III, IV and V). Hybridization was carried out over night at 68ºC, using 100ng/ml as the final probe concentration. Washing and detection of the membranes were performed according the recommendations of the DIG system (Roche). DIG-labeled nucleic acids were detected by chemiluminescence using X-ray films (GE, Healthcare) and a Molecular Imager Chemi-Doc system (Bio Rad).

In order to analyze the obtained results, an image processing algorithm was used. This software uses as references both the positive and negative controls present in the membranes and, calculates the probability of each dot being a positive signal. The exposure time in the Chemi-Doc system was adjusted to ensure that all dots were below pixel saturation (Albuquerque *et al.,*2011).

Table III- Layout of the first *Streptococcus agalactiae* membrane used in the dot-blot hybridization assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	SA7	SA253	SA329	SA335	SA254	SA322	SA332	SA344	SA202	SA203	SA204	SA7
B	TE	SA205	SA326	SA327	SA328	SA257	SA330	SA331	SA336	SA337	SA154	TE
C	SA194	SA195	SA196	SA197	SA323	SA324	SA325	SA258	SA319	SA334	SA346	SA255
D	SA320	SA321	SA333	SA345	SA318	SA343	SU112	SA38	SA275	SA276	SA277	SA221
E	SA222	SA283	SA284	SA285	SA286	SA67	SA68	SA232	SA233	SA270	SA271	SA272
F	SA243	SA244	SA295	SA35	SA223	SA296	SA249	SA250	SA251	SA252	SA307	SA308
G	TE	SA309	SA310	SU16	SA58	SA218	SA219	SA266	SA267	SA234	SA306	TE
H	SA7	SU113	SA56	SA245	SA246	SA247	SA248	**SA311**	SA312	SA313	SA314	SA7

SU112, SU16 and SU113- negative controls.

Table IV- Layout of the second *Streptococcus agalactiae* membrane used in dot blot hybridization assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	SA7	SA207	SA260	SA224	SA225	SA226	SA227	SA302	SA303	SA304	SA305	SA7
B	TE	SA50	SA237	SA238	SA239	SA240	SU52	SU58	SA57	SA300	SU16	TE
C	TE	SA59	SA241	SA242	SA280	SA281	SA282	SU90	SA43	SA214	SA301	TE
D	SA7	TE	TE	TE	TE	TE	TE	TE	TE	TE	TE	SA7

SU52, SU58, SU16 and SU90- negative controls.

Table V- Layout of the *Streptococcus uberis* membrane in the dot blot hybridization assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	SU3	SU57	SU91	SU114	SU52	SU53	SU58	SU59	SU16	SU112	SU90	SU3
B	TE	SU113	SA283	SU76	SU80	SU72	SU73	SU89	SU70	SU86	SU63	TE
C	SU64	SU65	SU82	SU83	SU67	SU103	SA284	SU40	SU62	SU79	SU99	SU66
D	TE	SU81	SU105	SU45	SU101	SU42	SU43	SU69	SU85	SU48	SU49	TE
E	SU3	SU68	SU87	SU88	SU104	SA285	SU60	SU61	SU84	SU98	SA286	SU3

.SA283, SA284, SA285 and SA286- negative controls.

5. CAMP test

The CAMP test is a presumptive identification test of *S. agalactiae* (Lancefield group B). (Phillps *et al*., 1980, Ratner *et al*., 1986). 5% sheep blood agar was used as cultured medium. This test consists in a synergistic lysis of sheep erythrocytes between *Staphylococcus aureus* and a protein from group B *S. agalactiae,* the CAMP factor (pathogenicity factor) (Gase *et al.*,1999). A positive result is verified by the presence of a halo which confirms β-hemolysis. To perform this assay, a strain of *S. aureus* LMG 8224 was placed in the center of a culture plate, and four strains of *S.agalactiae* two on each side were placed 3 mm perpendicularly to the strain, without direct contact. *Streptococcus uberis, Bacillus subtillis* and *Klebsiella pneumonia* were used as negative controls. The plates were incubated overnight at 37ºC. A total of seventy two strains were tested in eighteen petri dishes (Table VI).

Only one strain (1.4%) presented a negative result (SA311) (Figure 1). Sequencing of the 16S rRNA gene allowed identifying SA311 as *Staphylococcus epidermidis.* (Weiseburg *et al*., 1991) This analysis showed that this strain was misidentified as *S.agalactiae* by the VITEK system.

Table VI- Scheme used for plating the strains in petri dishes.

Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8	Plate 9
SA214	**SA232**	**SA223**	**SA211**	**SA246**	**SA238**	**SA43**	**SA190**	**SA194**
SA215	**SA233**	**SA252**	**SA94**	**SA247**	**SA239**	**SA59**	**SA191**	**SA195**
SA216	**SA207**	**SA218**	**SA56**	**SA50**	**SA241**	**SA68**	**SA192**	**SA196**
SA217	**SA243**	**SA219**	**SA245**	**SA237**	**SA242**	**SA93**	**SA193**	**SA197**

Plate 10	Plate 11	Plate 12	Plate 13	Plate 14	Plate 15	Plate 16	Plate 17	Plate 18
SA198	**SA202**	**SA208**	**SA300**	**SA304**	**SA308**	**SA312**	**SA316**	**SA321**
SA199	**SA203**	**SA297**	**SA301**	**SA305**	**SA309**	**SA313**	**SA317**	**SA322**
SA200	**SA204**	**SA298**	**SA302**	**SA306**	**SA310**	**SA314**	**SA318**	**SA323**
SA201	**SA205**	**SA299**	**SA303**	**SA307**	**SA311**	**SA315**	**SA319**	**SA324**

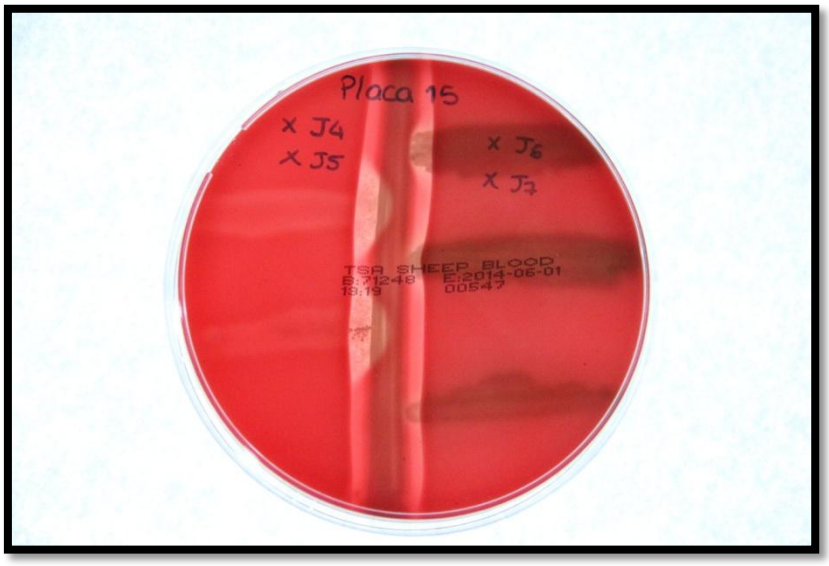

Figure 1 – Example of the CAMP test plates. The central strain is Staphylococcus aureus LMG 8224, and the arrow head shaped hemolytic halo associated to the Streptococcus agalactiae isolates is indicative of a positive result. The isolated cultured in the right side of the petri plates middle is a negative control, and the strain in the right bottom is a CAMP factor negative (XJ7- corresponding to isolate SA311).

RESULTS

1. Mastitis frequency in herds

Milk quality programs have been increasingly implemented on farms in order to reduce clinical mastitis and decrease the CCS in milk. To accomplish this task, veterinarians are expected to visit frequently the farms, in order to evaluate the general health state of cows and to collect milk and bulk tank samples to search for pathogenic agents causing clinical and sub-clinical infections.

In this study, several visits to the four selected herds (A, E, F and Z) were carried out in order to select cows diagnosed with mastitis caused by *S.agalactiae* and *S. uberis* in consecutive visits. The results obtained from this study showed that, from a total of 154 isolates, only 3.9% were responsible for clinical mastitis. Additionally, it was shown, that the average somatic cell count was higher in farms prevalent for *S.uberis*, than in farms where mastitis was predominantly caused by *S.agalactiae.* Regarding data analysis, *S.agalactiae* infections presented both the highest and the lowest SCC. The lowest SCC are easily unnoticed, as the threshold of detection of mastitis is 200.000 somatic cells per milliliter (Table VIII). Concerning

bacterial virulence patterns, it was possible to observe that for each pathogenic agent, both *S.agalactiae* and *S.uberis*, there are some virulence patterns that are more common amongst cows, and that there are some virulence patterns that cause an elevated count of SCC, which translates in a more severe mammary gland infection (Table IX). From the 4 herds studied, 37 cows were diagnosed with mastitis in repeated visits, from which, 22 cows infected with *S.agalactiae* and 15 with *S.uberis* (Table VII).

Table VII- Grid showing the selected cows for this study.

Herds	Visits	Months	Cows	Samples collected		Locality	Agent
				All	Quarters		
A	3	Dec/13	14	29	1	Barcelos	*S. agalactiae*
		jan/14		33	0		
		mai/14		not valued	not valued		
E	5	nov/13	4	2	0	Barcelos	*S. uberis*
		Dec/13		3	0		
		jan/14		0	0		
		Feb/14		2	0		
		Apr/14		0	3		
F	4	Dec/13	8	8	5	Póvoa do Varzim	*S. agalactiae*
		jan/14		13	1		
		mar/14		6	0		
		Apr/14		0	4		
Z	4	nov/13	11	6	0	Maia	*S. uberis*
		jan/14		13	1		
		Feb/14		11	0		
		Apr/14		0	6		

nv- not valued

Table VIII- Data analysis of simple and composed samples.

Agent	Statistics	AD	AE	PD	PE	ALL
S. uberis	Mean	1416,5	5945,5	1568,7	883,2	1071,3
	St. Deviation	1206,69	4917,64	1691,27	372,10	889,80
	Maximum	3782	12914	5498	1156	3457
	Minimum	172	223	165	138	135
S. agalactiae	Mean	2121,3	435,7	1496,4	1420,2	1175,6
	St. Deviation	3128,13	394,97	1921,68	1315,35	2441,19
	Maximum	10077	1575	5725	4913	9153
	Minimum	106	104	85	60	50

Table IX- Data analysis showing strain code, number of isolates and respective mean of SCC.

Streptococcus uberis			Streptococcus agalactiae		
Strain Code	Counting	Mean of SCC	Strain Code	Counting	Mean of SCC
1	1	5147	12	1	11200
2	37	1952	3	2	3138
3	4	1619	7	1	1306
6	1	374	1	94	1276
4	3	262	4	1	941
5	1	230	9	1	828
			13	1	427
			11	1	355
			8	2	282
			10	2	173
			6	1	50
Total	47	1848	Total	107	1329,4

2. Preliminary tests

In order to assess the efficiency of taxonomic DNA markers for rapid identification of mastitis isolates, all the strains collected in this work were tested by dot blot assays.

Results corresponding to markers- V1, V2,V3 and SU showed that all these markers gave positive hybridization signals with all *S.uberis* strains tested. Marker NU3, which is specific for *S.uberis*, as expected, was negative for the tested *S.agalactiae* strains. Due to inconsistent results obtained with marker F1, a new F1 probe obtained from a *S. agalactiae* strain (SAA9) was labeled and tested. This new labelled taxonomic marker previously reported to be genus-specific for *Streptococcus*, provided positive results with all tested strains (data not shown).

3. Assays with selected strains

3.1- Taxonomic markers

Concerning the taxonomic and virulence analyses of all selected strains, three types of dot-blot membranes were made, two for *S.agalactiae*, due to its higher number of isolates, and one for *S.uberis*. The markers selected for testing with the *S.agalactiae* membranes were F1SAA9, A1, CAMP, *fbsB*, FO3 and FO1. For *S.uberis* membrane F1SAA9, CAMP, NU1, *pauA*, *sua* and *ermB* were the markers used for the hybridization assays.

Regarding the probability values of hybridization outputted by the image analysis software used, a color code was employed to evaluate the obtained results 0 to 0.25 - low probability, represented in red, 0.25 to 0.75 -average probability, represented in yellow and 0.75 to 1.00-high probability, represented in green.

A total of 110 isolates of *S.agalactiae* and 48 isolates of *S.uberis* were tested. For *S.agalactiae* membranes, the *S.uberis* isolates SU112, SU113, SU16, SU52, SU58 and SU90

were used as negative controls (these strains were also tested in *S.uberis* membranes). For *S.uberis* membrane, the *S.agalactiae* isolates used negative controls were SA283, SA284, SA285 and SA286, which were also evaluated in *S.agalactiae* membranes.

A preliminary analysis of the obtained hybridization data revealed that the results obtained with three isolates previously identified by the VITEK system as *S.agalactiae* (SA332, SA272 and SA311) and one isolate identified as *S.uberis* (SU48), were indicative of a misidentification, as shown in Table X. In fact, 16S rRNA gene sequencing of the isolates, showed that these bacteria were incorrectly identified by the VITEK system and were withdrawn from further analysis.

Table X- Isolates with atypical results in Dot-Blot membranes, that after 16S gene sequencing analysis, were withdrawn from this study.

Streptococcus uberis membrane							
Strains	**ermB**	**sua**	**pau_A**	**NU1**	**CAMP**	**F1 (SAA9)**	**16S analysis**
SU48	0,04	0,00	0,00	0,00	0,00	0,00	*Enterococcus*
Streptococcus agalactiae membrane							
Strains	**FO3**	**FO1**	**fbsb**	**CAMP**	**A1**	**F1 (SAA9)**	**16S analysis**
SA332	0,03	0,00	0,00	0,00	0,00	0,99	*Streptococcus uberis*
SA272	0,10	0,16	0,06	0,01	0,03	1,00	*Streptococcus uberis*
SA311	0,01	0,06	0,00	0,01	0,04	0,01	*Staphilococcus epidermidis*

Regarding *Streptococcus* specific taxonomic marker F1 SAA9, dot blot hybridization showed a specificity of 99.1% (106/107) in *S.agalactiae* membranes, with only one isolate presenting a very low probability value of hybridization. However, when tested for marker A1, a taxonomic marker specific for this species, i.e. *S.agalactiae*, the result was a positive hybridization allowing to confirm the identity of this isolate.

Marker A1, had a specificity of 92.5% (99/107), with eight isolates, 7.5% (8/107), presenting an average hybridization probability.

In *S.uberis* membrane, the only taxonomic marker tested, F1 SAA9, present a specificity of 98% (46/47).

3.2- Virulence markers

A survey of the literature, allowed to select several virulence factors that seem to play an important role during mammary gland infections. In the present study, regions related to adhesion and invasion, toxin production, ability to growth in milk or in the environment and production of bacteriocins were selected for analysis.

In *S.agalactiae* membranes, concerning adhesion and invasion to the epithelium, the fibrinogen binding protein (*fbsB*) probe was selected. In *S.uberis* membrane the adhesion molecule (*Sua*) was tested.

Concerning toxin production, the CAMP factor was tested for both *Streptococcus* species.

The ability of *S.uberis* to grow in milk/ environment was assessed using the *pauA* factor probe, and in *S.agalactiae*, the fructose operons FO1 and FO3 were selected. For the study of bacteriocins, NU1, was chosen.

Table XI- Virulence factors for *S.agalactiae* used in this study.

Function and name	Gene	Reference
Adhesion - Fibrinogen binding protein	*fbsB*	Jacobsson *et al*., 2003
Ability to growth in milk/environment- Frutose operon	*fruD*	Richards *et al.,* 2011
Ability to growth in milk/environment- Frutose operon	*fruR*	Richards *et al.,* 2011
Toxin- CAMP factor	*cfb*	Chen *et al*., 2005

Table XII- Virulence factors for *S.uberis* used in this study.

Function and name	Gene	Reference
Adhesion and invasion- S.uberis adhesion molecule	*sua*	Almeida *et al.,* 2006
Ability to growth in milk/environment- Plasminogen activator	*pauA*	Rosey *et al*., 1999; Ward & Leight, 2002
Toxin- CAMP factor	*cfu*	Reinoso *et al.,* 2011
Bacteriocin- Nisin U	*nsu*	Wirawan *et al*., 2006

In *S.agalactiae* membranes, (Table XI), the results obtained with the fbsB probe revealed that this gene was present in 95.3% (102/107) of the strains. Three strains (2.8%) have an average probability to have the gene, and two isolates (1.9%) were negative suggesting that these isolates do not have the *fbsB* gene.

Results from the CAMP factor marker, showed that 95.3% (102/107) of the isolates were positive for this gene, four (3.7%) have an average probability and one isolate (0.9%) was negative for this gene.

Markers from fructose operons revealed that for FO1, 92.5% (99/107) of the tested isolates have the gene, four isolates (3.7%) have an average probability and four isolates (3.7%) were negative for the presence of FO1. For the markers FO3, 98.1% of the isolates studied (105/107) were positive for the presence of this gene with high probability values and 1.9% have an average probability.

Table XIII- Probability values of the results obtained from the Dot-Blot assays from *S.agalactiae* membranes

Strain	FO3	FO1	fbsb	CAMP	A1	F1SAA9	COW	TEAT	SCC	AGENTS	HC	DATE	CODE
SA253	1,00	1,00	1,00	1,00	0,99	0,98	19	ALL	253		AGX22	Dez.2013	1
SA329	1,00	1,00	1,00	1,00	0,82	0,99	19	AD	679	SCN	AGX22	Jan.2014	1
SA335	1,00	1,00	1,00	1,00	0,40	0,99	19	AD	2106		AGX22	Mar.2014	1
SA254	1,00	1,00	1,00	1,00	1,00	1,00	35	ALL	92		AGX22	Dez.2013	1
SA322	1,00	1,00	1,00	1,00	0,96	1,00	35	AE	243	SCN	AGX22	Jan.2014	1
SA344	1,00	1,00	0,98	0,98	0,45	1,00	35	ALL	180		AGX22	Abr.2014	1
SA202	1,00	0,97	0,30	0,98	0,34	1,00	45	AD	2666		AGX22	Dez.2013	3
SA203	1,00	1,00	0,70	1,00	0,99	1,00	45	PD	3609		AGX22	Dez.2013	3
SA204	1,00	1,00	0,98	1,00	0,61	1,00	45	AE	1575		AGX22	Dez.2013	1
SA205	0,88	0,98	**0,20**	0,92	0,78	0,85	45	PE	941		AGX22	Dez.2013	4
SA326	1,00	1,00	1,00	1,00	1,00	1,00	45	AD	835	STR	AGX22	Jan.2014	1
SA327	1,00	1,00	1,00	1,00	1,00	1,00	45	PD	1726		AGX22	Jan.2014	1
SA328	1,00	1,00	1,00	1,00	1,00	1,00	45	PE	2682	SCN	AGX22	Jan.2014	1
SA257	0,82	0,96	0,93	0,77	0,61	0,88	66	ALL	608		AGX22	Dez.2013	1
SA330	1,00	1,00	1,00	1,00	1,00	1,00	66	PD	263	SCN	AGX22	Jan.2014	1
SA331	1,00	1,00	1,00	1,00	1,00	1,00	66	PE	306		AGX22	Jan.2014	1
SA336	1,00	1,00	1,00	1,00	1,00	1,00	66	PD	510	SCN	AGX22	Mar.2014	1
SA337	1,00	1,00	1,00	1,00	1,00	1,00	66	PE	5935	SCN	AGX22	Mar.2014	1
SA154	1,00	1,00	1,00	1,00	0,83	1,00	74	ALL	271		AGX22	Abr.2013	1
SA194	1,00	1,00	1,00	1,00	1,00	1,00	74	AD	605		AGX22	Dez.2013	1
SA195	1,00	1,00	1,00	1,00	1,00	1,00	74	PD	1708		AGX22	Dez.2013	1
SA196	1,00	1,00	1,00	1,00	1,00	1,00	74	AE	1124		AGX22	Dez.2013	1
SA197	1,00	1,00	1,00	1,00	1,00	1,00	74	PE	800		AGX22	Dez.2013	1
SA323	1,00	1,00	1,00	1,00	1,00	1,00	74	AD	282	YST	AGX22	Jan.2014	1
SA324	1,00	1,00	1,00	0,96	1,00	1,00	74	PD	784		AGX22	Jan.2014	1
SA325	1,00	1,00	1,00	1,00	1,00	1,00	74	AE	565	SCN	AGX22	Jan.2014	1
SA258	0,97	1,00	1,00	0,97	1,00	0,90	77	ALL	222		AGX22	Dez.2013	1
SA319	1,00	1,00	1,00	1,00	1,00	1,00	77	AE	324		AGX22	Jan.2014	1
SA334	1,00	1,00	1,00	1,00	1,00	1,00	77	AE	251	SCN/ECO	AGX22	Mar.2014	1
SA346	1,00	1,00	1,00	1,00	1,00	1,00	77	ALL	197		AGX22	Abr.2014	1
SA255	1,00	1,00	1,00	1,00	1,00	1,00	478	ALL	8969		AGX22	Dez.2013	1
SA320	1,00	1,00	1,00	1,00	1,00	1,00	478	AE	341	SCN	AGX22	Jan.2014	1
SA321	1,00	1,00	1,00	1,00	1,00	1,00	478	PE	2545	SAR	AGX22	Jan.2014	1
SA333	1,00	1,00	1,00	1,00	1,00	1,00	478	AE	192	SCN	AGX22	Mar.2014	1
SA345	1,00	1,00	1,00	1,00	1,00	1,00	478	ALL	720		AGX22	Abr.2014	1
SA318	1,00	1,00	1,00	1,00	1,00	1,00	53	ALL	427	SCN	AGX22	Jan.2014	1
SA343	1,00	1,00	1,00	1,00	0,98	1,00	53	ALL	285		AGX22	Abr.2014	1
SA38	1,00	1,00	1,00	1,00	1,00	1,00	8	ALL	253		ASS74	Dez.2012	1
SA275	1,00	1,00	1,00	1,00	1,00	1,00	8	AD	10077		ASS74	Jan.2014	1
SA276	1,00	1,00	1,00	1,00	1,00	1,00	8	AE	214	STR	ASS74	Jan.2014	1
SA277	1,00	1,00	1,00	1,00	1,00	1,00	8	PE	444	STR/SCN	ASS74	Jan.2014	1
SA221	1,00	1,00	1,00	1,00	1,00	1,00	26	PD	949		ASS74	Dez.2013	1
SA222	1,00	1,00	1,00	1,00	1,00	1,00	26	PE	135		ASS74	Dez.2013	1
SA283	1,00	1,00	1,00	1,00	1,00	1,00	26	AD	501	SAR	ASS74	Jan.2014	1

SA284	1,00	1,00	1,00	1,00	1,00	1,00	26	PD	330		ASS74	Jan.2014	1
SA285	1,00	1,00	1,00	0,95	1,00	1,00	26	AE	223		ASS74	Jan.2014	1
SA286	1,00	1,00	1,00	1,00	1,00	1,00	26	PE	1393	SCN	ASS74	Jan.2014	1
SA67	1,00	1,00	1,00	1,00	1,00	1,00	29	AD	15719		ASS74	Dez.2012	1
SA68	1,00	1,00	1,00	1,00	1,00	1,00	29	ALL	9153		ASS74	Dez.2012	1
SA232	1,00	1,00	1,00	1,00	1,00	1,00	29	AE	896		ASS74	Dez.2013	1
SA233	1,00	1,00	1,00	1,00	1,00	1,00	29	PE	83		ASS74	Dez.2013	1
SA270	1,00	1,00	1,00	1,00	1,00	1,00	29	AD	57		ASS74	Jan.2014	1
SA271	1,00	1,00	1,00	1,00	1,00	1,00	29	PD	4888	STR	ASS74	Jan.2014	1
SA243	0,94	1,00	1,00	1,00	1,00	0,99	30	PD	201		ASS74	Dez.2013	1
SA244	1,00	1,00	1,00	1,00	1,00	1,00	30	PE	621		ASS74	Dez.2013	1
SA295	1,00	1,00	1,00	1,00	1,00	1,00	30	PE	2299		ASS74	Jan.2014	1
SA35	1,00	1,00	1,00	1,00	1,00	1,00	44	ALL	56		ASS74	Dez.2012	1
SA223	1,00	1,00	1,00	1,00	1,00	1,00	44	PE	6545		ASS74	Dez.2013	1
SA296	1,00	1,00	1,00	1,00	1,00	1,00	44	PE	3281		ASS74	Jan.2014	1
SA249	1,00	1,00	1,00	1,00	1,00	1,00	57	AD	616	SCN/YST	ASS74	Dez.2013	1
SA250	1,00	1,00	1,00	1,00	1,00	1,00	57	PD	248		ASS74	Dez.2013	1
SA251	1,00	1,00	1,00	1,00	1,00	1,00	57	AE	200		ASS74	Dez.2013	1
SA252	1,00	1,00	1,00	1,00	1,00	1,00	57	PE	1134		ASS74	Dez.2013	1
SA307	1,00	1,00	1,00	1,00	1,00	1,00	57	AD	2641	SCN	ASS74	Jan.2014	1
SA308	1,00	1,00	1,00	1,00	1,00	1,00	57	PD	341		ASS74	Jan.2014	1
SA309	1,00	1,00	1,00	1,00	1,00	1,00	57	AE	287		ASS74	Jan.2014	1
SA310	0,94	1,00	0,99	0,96	1,00	0,91	57	PE	1857	SCN	ASS74	Jan.2014	1
SA218	1,00	1,00	1,00	1,00	1,00	1,00	81	AE	410		ASS74	Dez.2013	1
SA219	1,00	1,00	1,00	1,00	1,00	1,00	81	PE	239		ASS74	Dez.2013	1
SA266	1,00	1,00	1,00	1,00	1,00	1,00	81	AE	810		ASS74	Jan.2014	1
SA267	1,00	1,00	1,00	1,00	1,00	1,00	81	PE	3637		ASS74	Jan.2014	1
SA234	1,00	1,00	1,00	1,00	1,00	1,00	121	PE	138		ASS74	Dez.2013	1
SA306	1,00	1,00	1,00	1,00	1,00	1,00	121	PE	558	SCN/STR	ASS74	Jan.2014	1
SA56	1,00	1,00	1,00	1,00	1,00	1,00	866	ALL	328		ASS74	Dez.2012	1
SA245	1,00	1,00	1,00	1,00	1,00	1,00	866	AD	106		ASS74	Dez.2013	1
SA246	1,00	1,00	1,00	1,00	1,00	1,00	866	PD	44		ASS74	Dez.2013	1
SA247	1,00	1,00	1,00	1,00	1,00	1,00	866	AE	115		ASS74	Dez.2013	1
SA248	1,00	1,00	1,00	1,00	1,00	1,00	866	PE	69	FUN	ASS74	Dez.2013	1
SA312	1,00	1,00	1,00	1,00	1,00	1,00	866	PD	126	SCN	ASS74	Jan.2014	1
SA313	1,00	1,00	1,00	1,00	1,00	1,00	866	AE	93	SCN	ASS74	Jan.2014	1
SA314	1,00	1,00	1,00	1,00	1,00	1,00	866	PE	50		ASS74	Jan.2014	1
SA207	0,76	**0,24**	0,73	0,87	0,32	0,75	714	ALL	50		ASS74	Dez.2013	6
SA260	1,00	0,98	0,98	1,00	0,97	1,00	714	AE	317		ASS74	Jan.2014	1
SA224	0,71	0,44	0,75	0,88	0,82	0,90	901	AD	1306		ASS74	Dez.2013	7
SA225	1,00	0,96	0,99	1,00	0,92	1,00	901	PD	492		ASS74	Dez.2013	1
SA226	0,98	0,94	0,98	1,00	0,97	0,93	901	AE	221		ASS74	Dez.2013	1
SA227	0,99	0,85	0,99	1,00	0,91	0,97	901	PE	1012		ASS74	Dez.2013	1
SA302	1,00	0,98	0,86	0,98	0,98	0,93	901	AD	136		ASS74	Jan.2014	1
SA303	1,00	0,99	0,98	0,99	0,63	1,00	901	PD	118		ASS74	Jan.2014	1
SA304	0,85	0,97	0,88	1,00	0,97	0,94	901	AE	74		ASS74	Jan.2014	1

SA305	0,95	0,93	0,95	0,97	0,41	0,99	901	PE	373	STR	ASS74	Jan.2014	1
SA50	1,00	0,99	1,00	1,00	0,98	1,00	916	ALL	1815		ASS74	Dez.2012	1
SA237	0,99	0,94	0,99	0,95	0,91	1,00	916	AD	1332		ASS74	Dez.2013	1
SA238	1,00	0,97	0,99	1,00	0,96	1,00	916	PD	1092		ASS74	Dez.2013	1
SA239	1,00	0,57	0,98	1,00	0,98	0,95	916	AE	323	ETB	ASS74	Dez.2013	8
SA240	1,00	**0,04**	**0,08**	0,29	0,94	**0,21**	916	PE	828		ASS74	Dez.2013	9
SA57	1,00	0,97	1,00	**0,02**	0,99	1,00	917	ALL	254		ASS74	Dez.2012	10
SA300	1,00	1,00	1,00	1,00	1,00	1,00	917	AD	114		ASS74	Jan.2014	1
SA59	1,00	1,00	1,00	1,00	0,99	1,00	919	ALL	132		ASS74	Dez.2012	1
SA241	1,00	1,00	1,00	1,00	0,96	1,00	919	PD	249		ASS74	Dez.2013	1
SA242	1,00	0,90	1,00	1,00	0,97	1,00	919	AE	242		ASS74	Dez.2013	1
SA280	1,00	**0,15**	1,00	1,00	1,00	1,00	919	AD	355	SCN/STR	ASS74	Jan.2014	11
SA281	1,00	**0,09**	1,00	**0,10**	1,00	1,00	919	PD	11200		ASS74	Jan.2014	12
SA282	0,99	0,40	1,00	0,97	0,98	1,00	919	AE	241	SCN	ASS74	Jan.2014	8
SA43	0,82	0,92	1,00	**0,04**	0,96	1,00	920	ALL	91		ASS74	Dez.2012	10
SA214	0,66	0,64	0,81	0,70	0,77	1,00	920	PD	427		ASS74	Dez.2013	13
SA301	0,96	0,98	1,00	1,00	0,99	1,00	920	AD	1268	SCN	ASS74	Jan.2014	1

SCN-*Estaphilococcus coagulase negativa*, STR- *Streptococcus,spp*, ETB- *Enterobacteriaceae*, FUN- Fungi, YST- Yeast, SAR- *Staphilococcus aureus,* ECO- *Escherichia coli*. AXG22- farm F, ASS74- farm A.

Concerning *S.uberis* membrane, (Table XIV), the *sua* marker is present in 95.7% (45/47) of the isolates, and two isolates (4.3%) have an average probability. Concerning bacteriocin production, NU1 was present in 89.4% (42/47) of the isolates and five strains (10.6%) do not have this gene.

Finally none of the *S.uberis* isolates (100%) presented the CAMP factor gene and the *pauA* gene was present in all strains (100%) evaluated.

Table XIV- Probability values of the results obtained from the Dot-Blot assays from *S.agalactiae* membranes

Strain	ermB	sua	pauA	NU1	CAMP	F1SAA9	COW	TEAT	SCC	AGENTS	HC	DATE	CODE
SU57	0,10	0,66	1,00	0,03	0,00	1,00	35	AD	5147		AS5N9	Dez.2013	1
SU91	0,91	0,98	1,00	0,21	0,00	1,00	35	AD	2417	ECO	AS5N9	Fev.2014	2
SU114	0,04	0,96	1,00	0,00	0,00	1,00	35	ALL	135		AS5N9	Abr.2014	3
SU52	0,88	0,96	1,00	1,00	0,00	1,00	65	AD	307		AS5N9	Nov.2013	4
SU53	0,72	0,86	1,00	1,00	0,00	1,00	65	AE	230		AS5N9	Nov.2013	5
SU58	0,16	0,69	0,99	1,00	0,00	1,00	65	AD	374		AS5N9	Dez.2013	6
SU59	0,89	0,97	0,98	1,00	0,00	1,00	65	AE	216		AS5N9	Dez.2013	4
SU 16	0,01	0,91	1,00	0,11	0,00	0,99	50	ALL	541		AS5N9	Jan.2013	3
SU112	0,24	0,98	1,00	0,11	0,00	1,00	50	ALL	302	SCN	AS5N9	Abr.2014	3
SU90	0,08	0,98	1,00	0,00	0,00	1,00	75	PD	5498		AS5N9	Fev.2014	3
SU113	0,97	1,00	1,00	0,07	0,00	1,00	75	ALL	3457	SCN	AS5N9	Abr.2014	2
SU76	0,98	1,00	1,00	0,04	0,00	1,00	8	AD	201		BG04N	Jan.2014	2
SU80	0,98	1,00	1,00	0,07	0,00	1,00	8	AD	142		BG04N	Fev.2014	2
SU72	0,99	1,00	1,00	0,09	0,00	1,00	18	PD	165		BG04N	Jan.2014	2
SU73	1,00	1,00	1,00	0,04	0,00	1,00	18	AE	1677		BG04N	Jan.2014	2
SU89	1,00	1,00	1,00	0,15	0,00	1,00	18	PE	1156	SCN	BG04N	Fev.2014	2

SU70	1,00	1,00	1,00	0,13	0,00	1,00	622	AD	295		BG04N	Jan.2014	2
SU86	0,99	1,00	1,00	0,11	0,00	1,00	622	AD	193		BG04N	Fev.2014	2
SU63	0,92	0,96	1,00	0,04	0,00	0,96	629	AD	456		BG04N	Jan.2014	2
SU64	0,95	0,95	0,97	0,02	0,00	1,00	629	PD	1309		BG04N	Jan.2014	2
SU65	0,85	0,93	0,98	0,03	0,00	1,00	629	AE	223		BG04N	Jan.2014	2
SU82	0,87	0,98	1,00	0,21	0,00	1,00	629	AD	1742		BG04N	Fev.2014	2
SU83	1,00	1,00	1,00	0,15	0,00	1,00	629	PD	179		BG04N	Fev.2014	2
SU67	0,89	1,00	1,00	0,03	0,01	1,00	811	AD	321		BG04N	Jan.2014	2
SU103	0,99	1,00	1,00	0,09	0,00	1,00	811	ALL	334		BG04N	Abr.2014	2
SU40	0,99	1,00	1,00	0,07	0,00	0,96	855	PD	1416		BG04N	Nov.2013	2
SU62	0,90	0,98	1,00	0,21	0,00	0,97	855	ALL	747		BG04N	Jan.2014	2
SU79	1,00	1,00	1,00	0,18	0,00	0,63	855	PD	731		BG04N	Fev.2014	2
SU99	0,91	1,00	1,00	0,18	0,00	1,00	855	ALL	385		BG04N	Abr.2014	2
SU66	1,00	1,00	1,00	0,11	0,00	1,00	901	AE	5297		BG04N	Jan.2014	2
SU81	1,00	1,00	1,00	0,18	0,00	1,00	901	AE	20531		BG04N	Fev.2014	2
SU105	1,00	1,00	1,00	0,28	0,00	1,00	901	ALL	511		BG04N	Abr.2014	2
SU45	1,00	1,00	1,00	1,00	0,00	1,00	902	PE	IM		BG04N	Nov.2013	4
SU101	0,96	0,99	1,00	0,04	0,00	1,00	902	ALL	1151		BG04N	Abr.2014	2
SU42	0,98	0,99	1,00	0,03	0,00	1,00	942	AD	519		BG04N	Nov.2013	2
SU43	1,00	0,98	1,00	0,05	0,00	0,98	942	AE	372	SCN	BG04N	Nov.2013	2
SU69	1,00	1,00	1,00	0,03	0,00	0,97	942	AD	3169		BG04N	Jan.2014	2
SU85	1,00	1,00	1,00	0,05	0,00	1,00	942	AD	2122		BG04N	Fev.2014	2
SU49	1,00	1,00	1,00	0,09	0,00	1,00	947	PE	173		BG04N	Nov.2013	2
SU68	0,99	1,00	1,00	0,18	0,00	1,00	947	PD	799		BG04N	Jan.2014	2
SU87	1,00	1,00	1,00	0,13	0,00	1,00	947	PD	562		BG04N	Fev.2014	2
SU88	1,00	1,00	1,00	0,02	0,00	1,00	947	PE	1748	SCN	BG04N	Fev.2014	2
SU104	1,00	1,00	1,00	0,05	0,06	1,00	947	ALL	1138		BG04N	Abr.2014	2
SU60	1,00	1,00	1,00	0,04	0,00	0,98	950	PD	450		BG04N	Jan.2014	2
SU61	1,00	1,00	1,00	0,04	0,00	0,93	950	AE	14223		BG04N	Jan.2014	2
SU84	1,00	1,00	1,00	0,11	0,00	1,00	950	AE	409		BG04N	Fev.2014	2
SU98	1,00	1,00	1,00	0,09	0,00	1,00	950	ALL	1519		BG04N	Abr.2014	2

SCN-*Estaphilococcus coagulase negative,* ECO- *Escherichia coli.* AS5N9- farm E, BG04N- farm Z.

3.3- Antibiotics resistance marker

In this study, the erythromycin resistance gene *ermB,* was analyzed in *S.uberis,* and the results revealed a positive hybridization of 85.1% (40/47) from all the collected isolates, a negative hybridization in 12.3% (6/47) of the isolates, and an average hybridization in only one (2.13%) *S.uberis* isolate.

Interestingly, all the isolates that which were hybridization negative for *ermB* gene, were obtained from only one farm, i.e. farm E, located in Barcelos.

4- Genetic variation

Due to the information collected about all the isolates obtained during the farm visits it was possible to trace the individual profiles of isolates present in each animal at a certain date. After evaluating the farms with animals mainly infected with *S.uberis*, it was possible to observe a high heterogeneity of isolates obtained from animals in farm E. For instance, the isolate SU57 obtained in December 2013 from animal 35 had a hybridization value of 0.66 for the sua marker, i.e. below the high probability values obtained for most of the other *S.uberis* isolates for this marker, including the isolate SU114 with a probability value of 0.96, obtained in April 2014 from the same animal. However, a different hybridization pattern was found for another isolate (SU91) obtained from the same animal in February 2014, which revealed positive hybridizations to markers *ermB*, *sua*, *pauA* and F1SAA9 and negative hybridizations for NU1 and CAMP markers.

Concerning animal 65 the two isolates (SU52 and SU53) obtained in the visit of November 2013 showed the same hybridization profile, while the two isolates (SU58 and SU59) obtained one month later, i.e. in December 2013 were different with the isolate SU59 showing the same hybridization result as the isolates SU52 and SU53 obtained one month earlier, suggesting that this isolate might belong to the same clonal lineage as the isolates SU52 and SU53. In animal 50, isolates with the same hybridization pattern were obtained in both visits. On the contrary, in animal 75, two different hybridization profiles were obtained for the two isolates obtained in different visits (Table XIV).

Concerning farm Z, the results were very different, showing a strong homogeneity of isolates as almost all the animals studied originates isolates with identical hybridization signals regardless the visit, which suggests bacteria with a resilient behavior. The following pattern was repeated for the isolates obtained from every animal, positive hybridization for *ermB*, *sua, pauA* and F1SAA9 markers and negative hybridization for NU1 and CAMP markers. The only exception was cow number 902 which presented two isolates, one with an hybridization pattern equal to the one mentioned above and the other, collected in November 2013 (SU45), showed positive hybridization for the NU1 marker (Table XIV).

Regarding farms mainly affected with *S.agalactiae*, in farm F the animals 19, 35, 53, 66, 74, 77 and 478 presented high homogeneity with positive hybridization for all markers tested. The only exception found was animal 45, from which four isolates (SA202, SA203, SA204 and SA205) were obtained in December 2013. Interestingly all these isolates had different hybridization probabilities for *fbsB* marker, with 0.30 for isolate SA202; 0.70 for isolate SA203; 0.90 for isolate SA204 and 0.20 for isolate SA205. These results seem to suggest that different *S.agalactiae* lineages were infecting simultaneously the same cow. However, one month later, in January 2014, the isolates obtained from three teats of this animal (cow 45), revealed an

identical values of hybridization probability, positive to all markers assayed, suggesting that the three teats were colonized by the same *S.agalactiae* lineage.

In farm A, the animals 8, 26, 29, 30, 44, 57, 81, 121 and 866 present high homogeneity between isolates, with positive hybridization for all markers in the visits carried out. Nevertheless, exceptions were noticed in five cows. In animal 714, isolate SA207 obtained in December 2013, presented a negative hybridization for the FO1 marker and hybridization probability of 0.73 for *fbsB.* In the January 2014, the isolate SA260 obtained from the same animal was positive for all markers. These results suggest that, this cow presented putatively two different *S.agalactiae* lineages at two different time points. In animal 901, during the December 2013 visit, all the teats were sampled and further analysis revealed that isolate SA224 had hybridization probability values for FO3 and FO1 markers of 0.71 and 0.44 respectively. The isolates from the remaining teats and the ones collected in January 2014 showed hybridization probabilities close to 1 for all virulence markers.

In animal 916, isolates SA50, SA237 and SA 238 had high hybridization signals for all markers, however SA239 presented an hybridization probability of 0.57 for the FO1 marker, and SA 240 revealed a negative hybridization for FO1 and *fbsB* markers and a weak hybridization probabilty of 0.29 for CAMP probe. This data suggest that in the same visit (December 2013) this animal was infected by three different *S.agalactiae* lineages. Animal number 917 revealed two distinct isolates in December 2012 (SA57) and in January 2014 (SA300). While the isolate SA57 was positive for all markers, with the exception for CAMP factor, the isolate SA300 was also positive for CAMP.

In animal 919, strains SA59, SA241 and SA242 were positive to all markers, but in the visit of January 2014, the isolates obtained were different, with SA280 negative for marker FO1; SA281 negative for FO1 and CAMP and SA282 with a hybridization probability of 0.40 for FO1 marker. These results suggest that in January 2014 three different *S.agalactiae* were infecting this animal.

Finally, animal 920 presented three isolates with different hybridization patterns obtained at distinct dates: December 2012 (SA43); December 2013 (SA214); and January 2014 (SA301).

Concerning, the persistence of mastitis caused by *S.agalactiae*, the data gathered showed that, in farm A, 60% of the cows had an infected persistent quarter, and in farm F, 75% of the animals presented the same infected quarters (Table I- appendix). About persistence in *S.uberis* farms, in farm E, 50% of animals had persistent quarters, and in farm Z, 72.7% of the cows revealed persistence of mastitis (Table XV).

Table XV- CMT and isolates results from selected *Streptococcus uberis* cows.

Strain	ANIMAL	TEAT	TCM				Isolados				HC	DATE	CODE	TX
			AD	PD	AE	PE	AD	PD	AE	PE				
SU57	35	AD	1A	0	0	0	1				AS5N9	Dez.2013	1	
SU91	35	AD	-	-	-	-	2				AS5N9	Fev.2014	2	
SU114	35	TODOS	0	0	0	0	3	3	3	3	AS5N9	Abr.2014	3	
SU52	65	AD	2	S	2	2	4		5		AS5N9	Nov.2013	4	
SU58	65	AD	1	S	2	2	6		4		AS5N9	Dez.2013	6	
SU 16	50	TODOS	-	-	-	-	3	3	3	3	AS5N9	Jan.2013	3	
SU112	50	TODOS	0	0	1	0	3	3	3	3	AS5N9	Abr.2014	3	SY (Fev e Mar)
SU90	75	PD	-	-	-	-		3			AS5N9	Fev.2014	3	SY /YO/VO
SU113	75	TODOS	0	2	0	0	2	2	2	2	AS5N9	Abr.2014	2	
SU76	8	AD	1	1	0	0	2				BG04N	Jan.2014	2	
SU80	8	AD	0	0	0	0	2				BG04N	Fev.2014	2	
SU72	18	PD	1	0	2	0		2	2		BG04N	Jan.2014	2	
SU89	18	PE	2	2	2	2				2	BG04N	Fev.2014	2	
SU70	622	AD	2	0	2	0	2				BG04N	Jan.2014	2	
SU86	622	AD	1	0	0	S	2				BG04N	Fev.2014	2	
	622	TODOS	1	0	2	S					BG04N	Abr.2014		
SU63	629	AD	2	1	0	2	2	2	2		BG04N	Jan.2014	2	
SU82	629	AD	2	0	0	2	2	2			BG04N	Fev.2014	2	
	629	TODOS	2	1	1	2					BG04N	Abr.2014		
SU67	811	AD	2	0	0	2	2				BG04N	Jan.2014	2	
SU103	811	TODOS	2	1	2	1	2	2	2	2	BG04N	Abr.2014	2	
SU40	855	PD	2	2	1	0		2			BG04N	Nov.2013	2	
SU62	855	TODOS	2	2	2	0	2	2	2	2	BG04N	Jan.2014	2	
SU79	855	PD	0	0	0	0		2			BG04N	Fev.2014	2	
SU99	855	TODOS	2	2	S	1	2	2	2	2	BG04N	Abr.2014	2	
SU66	901	AE	0	0	2	0			2		BG04N	Jan.2014	2	
SU81	901	AE	0	0	1B	0			2		BG04N	Fev.2014	2	
SU105	901	TODOS	1	1	2	1	2	2	2	2	BG04N	Abr.2014	2	
SU45	902	PE	1	2	0	2				4	BG04N	Nov.2013	4	
	902	PE	2	2	2	1					BG04N	Jan.2014		
SU101	902	TODOS	2	2	2	2	2	2	2	2	BG04N	Abr.2014	2	
SU42	942	AD	2	2	2	2	2		2		BG04N	Nov.2013	2	
SU69	942	AD	2	2	2	2	2				BG04N	Jan.2014	2	
SU85	942	AD	2	2	2	2	2				BG04N	Fev.2014	2	
SU49	947	PE	0	2	0	2				2	BG04N	Nov.2013	2	
SU68	947	PD	0	0	1	2		2			BG04N	Jan.2014	2	
SU87	947	PD	0	2	1	2		2		2	BG04N	Fev.2014	2	
SU104	947	TODOS	2	2	2	2	2	2	2	2	BG04N	Abr.2014	2	
SU60	950	PD	0	1A	0	1		2	2		BG04N	Jan.2014	2	
SU84	950	AE	2	2	2	1			2		BG04N	Fev.2014	2	
SU98	950	TODOS	1	2	1	1	2	2	2	2	BG04N	Abr.2014	2	

SY- Synulox, YO- Yodimaspen, VO- Voren.

5- Search for pathogens in cow`s environment

In order to search for pathogens in cow's environment, swabs were performed in the teat cups, milkers' hands and sawdust in all the farms visited. The results are shown in Table XIV. None of isolates obtained were identified as *S.agalactiae* or *S.uberis*, however, the pathogens detected could act as a gateway for those mastitis causative pathogens.

Table XIV- Results from swabs collected in farms A *(S.agalactiae*) and Z (*S.uberis*).

Herd	Date	Local	Bacteriology 1	Bacteriology 2	Bacteriology 3
A	jan/14	MILKER HANDS 1 BM	Sta.aureus/intermedi	Streptococcus spp	Escherichia coli
A	jan/14	MILKER HANDS 2 BM	Negative cultures	-	-
A	jan/14	TEAT CUP 1 LS BM	Estaf.coag.neg	Streptococcus spp	Escherichia coli
A	jan/14	TEAT CUP 9 LS BM	Sta.aureus/intermedi	Streptococcus spp	Escherichia coli
A	jan/14	TEAT CUP 11 LS BM	Sta.aureus/intermedi	Streptococcus spp	Escherichia coli
A	jan/14	TEAT CUP 1 RS BM	Sta.aureus/intermedi	Streptococcus spp	Escherichia coli
A	jan/14	TEAT CUP 6 RS BM	Sta.aureus/intermedi	Streptococcus spp	Escherichia coli
A	jan/14	TEAT CUP 1 RS AM	Sta.aureus/intermedi	Estaf.coag.neg	Escherichia coli
A	jan/14	TEAT CUP 6 RS AM	Sta.aureus/intermedi	Streptococcus spp	Escherichia coli
A	jan/14	MILKER HANDS AM	Streptococcus spp	Bacillus spp	-
Z	Feb /14	MILKER HANDS 1 BM	Estaf.coag.neg	-	-
Z	Feb /14	MILKER HANDS 2 BM	Streptococcus spp	Estaf.coag.neg	-
Z	Feb /14	TEAT CUP 1 RS BM	Negative cultures	-	-
Z	Feb /14	TEAT CUP 3 RS BM	Negative cultures	-	-
Z	Feb /14	TEAT CUP 7 RS BM	Negative cultures	-	-
Z	Feb /14	TEAT CUP 2 LS BM	Negative cultures	-	-
Z	Feb /14	TEAT CUP 1 RS AM	Streptococcus spp	Estaf.coag.neg	Enterococcus spp
Z	Feb /14	TEAT CUP 3 RS AM	Streptococcus spp	Estaf.coag.neg	Bacillus spp
Z	Feb /14	TEAT CUP 7 RS AM	Enterococcus spp	Estaf.coag.neg	-
Z	Feb /14	TEAT CUP 2 LS AM	Estaf.coag.neg	Streptococcus spp	-
Z	Feb /14	MILKER HANDS 1 AM	Enterococcus spp	Estaf.coag.neg	Streptococcus spp
Z	Feb /14	MILKER HANDS 2 AM	Estaf.coag.neg	-	-
Z	Feb /14	UNUSED SAWDUST	Leveduras	-	-
Z	Feb /14	LACTATION COWS SAWDUST	Streptococcus spp	Estaf.coag.neg	-
Z	Feb /14	DRY COWS SAWDUST	Sta.aureus/intermedi	Streptococcus spp	Fungos

RS- Right side; LS- Left side; BM- Before milking; AM- After milking

DISCUSSION

1. Mastitis in herds

Despite all efforts undertaken by farmers to apply good practices in management and control of mammary infections, caused by pathogens like *S.agalactiae* and *S.uberis* in herds, in this work it was possible to determine that there is still a high prevalence of these pathogens in those farms.

In order to better understand outbreaks of *S.agalactiae* and *S.uberis* it is important to conduct epidemiological studies taking into account, several other factors, namely, cow´s genetics, bacterial virulence factors and specific mastitis control programs.

In this work, the genotypic properties of numerous strains isolated from cows of four dairy farms in monthly visits, as well as clinical information regarding these same cows were evaluated, aiming to define bacterial virulence characteristics, strategies for host invasion, and persistence in herds.

The data collected in this study showed that the mean SCC is higher in infections caused by *S.uberis* isolates, suggesting that this species could cause a more severe mammary gland inflammation, which requires better therapeutic care to control the infection and the spreading to other animals. However, *S.agalactiae* isolates have a wide range of infection, i.e. the highest and the lowest score of SCC, and for that reason clinical signs could be very different, ranging from mild or severe mastitis. Therefore, veterinarians should be aware of this variation in order to better plan the therapeutic control. Regarding genotypic properties of the isolates, we were able to observe that, *S.uberis* isolates that presented code 2 (positive signal hybridization for the sua and pauA markers and negative signal hybridization for the ermB, NU1 and CAMP markers), were the most commonly found in herds. On the other hand, code 1 *S.uberis* isolates presented a higher SCC, suggesting that this genotype could be more virulent than the others. Nevertheless, the most common genotype that was found did not present a very high SCC, and can be easily controlled with prophylactic measures. Concerning *S. agalactiae* isolates, the most common genotype is represented by the code 1 (positive signal hybridization with all markers), which have an average SCC that could easily decrease with appropriate drugs. The only isolate that presented a very high SCC is represented by code 12; suggesting that this type of strain could be very virulent for the mammary gland. Isolates with codes 10 and 6 presented a very low SCC and easily pass unnoticed under the threshold of mastitis detection. For this reason, veterinarians should not discard samples with these SCC values and should always perform a thorough analysis of available samples.

2. DNA- based identification of pathogens

The taxa-specific markers F1SAA9 and A1 correctly identified all *Streptococcaceae* and *S.agalactiae*, respectively. In general the results presented in this work, reinforce the good stability and consistency of these markers to be used in identification of *Streptococcaceae*, *S.agalactiae* and *S.uberis*.

Regarding the genes coding for adherence and invasion traits of bacteria to the host epithelial cells, the *fbsB* gene in *S.agalactiae* and the *sua* gene in *S.uberis*, the results obtained in this study showed that about 97.2% of the *S.uberis* isolates have a strong probability to

contain the *sua* gene. These results differ from other studies, showing that *sua* was not present in 20% of *S.uberis* strains isolated from cows with mastitis (Reinoso et al, 2011). The high presence of this *sua* in *S.uberis* isolates, may suggest that this gene plays an important role in establishing infection.

The fructose operons, *fruD* and *fruR,* were present in *S.agalactiae* in a rate of 98.1% and 92.5%, respectively. The ability to use fructose to obtain carbon sources is important for this bacteria to survive outside the mammary gland and, therefore, the presence of these markers, might be considered to confer an adaptative advantage to the dissemination of the infection.

Another operon that may provide competitive advantages during mastitis infections is the nisin U operon. In this study, this marker was present in 89.4% of the *S.uberis* strains evaluated. These results show that this bacteriocin might confer dominance to *S.uberis* when competing with other species (Pryor et al., 2009). Interestingly these results contrast to previous research where the presence of genes from the nisin U operon was limited to a few strains (Wirawan et al., 2006).

Regarding the ability to growth in milk, *pauA* was evaluated in *S.uberis* isolates, and all the strains presented a positive hybridization signal in dot blot assays. The presence of this gene is acknowledge to confer an advantage for this species to persist in the mammary glands, despite the flushing effect of milk during milking.

In relation to toxin production the CAMP factor was tested for both *S.agalactiae* and *S.uberis.*

The dot blot assays, revealed that none of the tested *S.uberis* isolates presented this gene, whereas, 95.3% of *S.agalactiae* isolates presented this gene. It is worth mentioning that CAMP factor assay is used as a presumptive identification of *S.agalactiae,* therefore, it is not surprising that the majority of *S.agalactiae* isolates were positive for this marker, contrary to what was observed for the *S.uberis* isolates.

The *ermB* antibiotic resistance coding gene was observed to be present in 85.1% of all *S.uberis* isolates studied. Besides the therapeutic informative value, these results confirm the spread and emergence of the macrolide resistance in the environment.

Briefly, altogether this study emphasizes the presence of diverse *S.agalactiae* and *S.uberis* genotypes associated with bovine mammary gland infections. The results further suggest the existence of isolates characterized by different virulence patterns, some of which obtained from the same animal at different time points. The high frequency of these virulence factors in the dozens of streptococci isolates analyzed suggest that bacteria possessing these genomic traits, are more able to cause infection and persist in environment, and therefore more likely to successfully cause mastitis infections. The genetic variability in farms mainly affected with *S.uberis* is higher in farm E, and in farms mainly affected with *S.agalactiae* is higher in farm

A. These differences may be due to environment features and cow`s factors which might worth considering in future studies.

3. Future perspectives

The results obtained in this research should be analyzed with caution because, despite the reasonable number of bacterial isolates collected, a larger timeframe of the study would be important to obtain more isolates from the same cow across a longer period, to better understand bacterial adaptation and evolution.

Nevertheless, regardless the inherent limitations, this study provide valuable information comprising epidemiological and molecular data, of these mastitis pathogens, which may contribute to improve treatment and containment measures. In addition this study contributed to disclose other research questions that will be important to address in future research, namely:

- Continue to collect at different time points samples from cows that were previously diagnosed with clinical or subclinical mastitis caused by *S.agalactiae* and *S.uberis* carriers, in order to determine detailed an epidemiological profiles. The cows monitored in this study can be a starting point.

- Investigate more genes coding for other virulence factors and antimicrobial resistance.

- Infer the geographical, temporal and cow`s housing patterns of individual cows infected with these streptococci agents, and correlate them with bacteria genomic traits.

- Gather as much as possible clinical information from infected cows, regarding their general health state, severity, persistence and antibiotics used and correlate this information with resistance to treatment, and bacterial properties.

- Standardize all the information in a database, in order to make it easily accessible and understandable to additional epidemiological and molecular studies.

APPENDIX

1. References

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2. Table

Table I- Appendix- CMT and isolates results from selected *Streptococcus agalactiae* cows.

Strain	ANIMAL	TEAT	CMT				Isolates				HC	DATE	TX
			AD	PD	AE	PE	AD	PD	AE	PE			
SA253	19	TODOS	2	0	0	0	1	1	1	1	AGX22	dez/13	
SA329	19	AD	1	0	0	0	1				AGX22	jan/14	
SA335	19	AD	2	2	0	0	1				AGX22	mar/14	
SA254	35	TODOS	0	0	0	0	1	1	1	1	AGX22	dez/14	
SA322	35	AE	S	2	0	0			1		AGX22	jan/14	
SA344	35	TODOS	S	0	2	1	3	3	3	3	AGX22	abr/14	
SA202	45	AD	1	2	2	2	3	3	1	4	AGX22	dez/13	
SA326	45	AD	1	2	0	2	1	1		1	AGX22	jan/14	
SA257	66	TODOS	0	2	S	1	1	1	1	1	AGX22	dez/13	
SA330	66	PD	0	1	S	0		1		1	AGX22	jan/14	
SA336	66	PD	2	2	S	2		1		1	AGX22	mar/14	PM
	66	TODOS	2	2	S	2					AGX22	abr/14	
SA154	74	TODOS	-	-	-	-	1	1	1	1	AGX22	abr/13	
SA194	74	AD	2	2	2	2	1	1	1	1	AGX22	dez/13	
SA323	74	AD	2	2	2	S	1	1	1	1	AGX22	jan/14	
SA258	77	TODOS	0	0	0	2	1	1	1	1	AGX22	dez/13	
SA319	77	AE	0	0	1	2			1		AGX22	jan/14	
SA334	77	AE	0	0	0	1			1		AGX22	mar/14	
SA346	77	TODOS	0	0	0	2	1	1	1	1	AGX22	abr/14	
SA255	478	TODOS	0	1	0	2	1	1	1	1	AGX22	dez/13	
SA320	478	AE	2	0	0	2			1	1	AGX22	jan/14	
SA333	478	AE	0	1	0	0			1		AGX22	mar/14	
SA345	478	TODOS	0	0	2	0	1	1	1	1	AGX22	abr/14	
SA318	53	TODOS	0	0	2	2	1	1	1	1	AGX22	jan/14	OE
SA343	53	TODOS	0	0	1	1	1	1	1	1	AGX22	abr/14	

SA38	8		2	2	1	2	1	1	1	1	ASS74	dez/12
SA275	8	AD	2	2	0	2	1		1	1	ASS74	jan/14
cont	8	TODOS	0	2	0	0					ASS74	abr/14
SA221	26	PD	0	2	0	2		1		1	ASS74	dez/13
SA283	26	AD	1	2	1	2	1	1	1	1	ASS74	jan/14
SA67	29		M	2	2	1	1	1	1	1	ASS74	dez/12
SA233	29	PE	0	2	0	0			1	1	ASS74	dez/13
SA270	29	AD	0	2	1A	0	1	1			ASS74	jan/14
SA243	30	PD	S	2	2	2		1		1	ASS74	dez/13
SA295	30	PE	S	2	2	2				1	ASS74	jan/14
SA35	44	TODOS	M	0	0	0	1	1	1	1	ASS74	dez/12
SA223	44	PE	1	0	0	2				1	ASS74	dez/13
SA296	44	PE	0	0	0	2				1	ASS74	jan/14
SA249	57	AD	2	2	2	2	1	1	1	1	ASS74	dez/13
SA307	57	AD	2	2	2	2	1	1	1	1	ASS74	jan/14
SA218	81	AE	0	0	0	1			1	1	ASS74	dez/13
SA266	81	AE	0	0	2	2			1	1	ASS74	jan/14
SA234	121	PE	0	0	0	2				1	ASS74	dez/13
SA306	121	PE	0	0	0	2				1	ASS74	jan/14
SA56	866	TODOS	0	2	0	0	1	1	1	1	ASS74	dez/12
SA245	866	AD	0	0	0	0	1	1	1	1	ASS74	dez/13
SA311	866	AD	0	1	0	0		1	1	1	ASS74	jan/14
SA207	714	TODOS	0	0	0	0	6	6	6	6	ASS74	dez/13
SA260	714	AE	0	0	2	1			1		ASS74	jan/14
SA224	901	AD	2	2	0	2	7	1	1	1	ASS74	dez/13
SA302	901	AD	0	1	0	0	1	1	1	1	ASS74	jan/14
SA50	916	TODOS	2	1	1	1	1	1	1	1	ASS74	dez/12
SA237	916	AD	2	2	1	2	1	1	8	9	ASS74	dez/13
SA57	917	TODOS	0	0	0	0	10	10	10	10	ASS74	dez/12
SA300	917	AD	1B	0	0	0	1				ASS74	jan/14
SA59	919	TODOS	0	0	0	0	1	1	1	1	ASS74	dez/12
SA241	919	PD	0	0	1	1		1	1		ASS74	dez/13
SA280	919	AD	2	2	0	1	11	12	8		ASS74	jan/14
SA43	920	TODOS	0	0	0	0	10	10	10	10	ASS74	dez/12
SA214	920	PD	0	2	2	2		13			ASS74	dez/13
SA301	920	AD	0	2	0	0	10				ASS74	jan/14

PM- Penicilina Mista; OE- Orbenin Extra

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